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(54) Title: ENERGY TRANSFER DYES

(57) Abstract

A novel class of energy transfer dyes, their preparation, and their use as labels in biological systems is disclosed. The dyes are preferably in the form of cassettes which enable their attachment to a variety of biological materials. The dyes and the reagents that can be made from them offer a wide variety of fluorescent labels with large Stokes' shifts enabling their use in a variety of fluorescence applications over a wide range of the visible spectrum.

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DESCRIPTION

ENERGY TRANSFER DYES

Field of the Invention

The present invention relates to a novel class of energy transfer dyes, their preparation and their use as labels in biological systems.

Background of the Invention

The following describes certain relevant art, none of which is admitted to be prior art to the appended claims.

UK Patent No. 2301 833 B discloses, *inter alia*, that complexes including:

i) a first fluorochrome having first absorption and emission spectra;

ii) a second fluorochrome having second absorption and emission spectra, the wavelength of the emission maximum of the second fluorochrome being longer than the wavelength of the emission maximum of the first fluorochrome, and a portion of the absorption spectrum of the second fluorochrome overlapping a portion of the emission spectrum of the first fluorochrome;

iii) at least one linker group chosen from the group consisting of alkyl chains containing from 1 to 20 carbon atoms, which may optionally include from 1 to 8 oxygen atoms as polyether linkages, or from 1 to 8 nitrogen atoms as polyamine linkages, or from 1 to 4 CO-NH groups as polyamide linkages and up to 2 bicyclo[2,2,2]octyl groups, for covalently attaching the first and second fluorochromes for transfer of resonance energy transfer between the first and second fluorochromes; and

iv) at least one target bonding group capable of forming a covalent bond with a target compound;

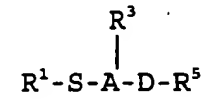
wherein at least one of the first or second fluorochromes is a cyanine dye and the combined molecular weight of the first and second fluorochromes and the linker group is less than about 20,000 Daltons, are energy transfer dyes which may be attached to biological systems. The fluorescent nature of the dyes enables them to monitor processes in which the biological systems are involved. They are of use in sequencing and in nucleic

acid detection.

Summary of the Invention

It has now been found that a novel class of energy transfer dyes are of use in labeling materials involved in sequencing reactions and other applications. The dyes are preferably in the form of "cassettes" which enable their attachment to a variety of biological materials. A cassette includes a covalently linked structure or complex with at least two fluorescent dye moieties, a linker group, and preferably a reactive group for attaching the complex to a biological material or other target material. The reactive group is chosen to be suitable for forming a covalent linkage with a functional group on a particular target material. The dyes are selected so that the emission spectrum of one dye overlaps the absorption spectrum of a second dye, so that energy transfer can occur between the dyes.

Accordingly, the present invention provides an energy transfer dye of the formula (I):



(I)

where R^1 is a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 is a second dye that is suitable as a donor or acceptor in an energy transfer arrangement with the first dye;

A comprises a chain that contains 5,6,7,8,9,10,11,12,13,14,15,16,17,18,19 or 20 linearly linked atoms selected from carbon, nitrogen and oxygen. The chain may optionally be substituted, if desired, with groups as known to those skilled in the art which do not prevent energy transfer, for example, by $C_{1,2,3\ or4}$ linear or branched alkyl or phenyl, optionally substituted with 1,2,3, or 4 substituents independently selected from OH, halo, methyl or ethyl) groups). Preferably A is a chain of linearly linked atoms;

R^3 is a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material, e.g., a biological material as noted above; and

D comprises an atom or group for attaching R^5 to the linker chain A, in which the covalent linkage between A and R^5 does not include a sulphur atom. Also, preferably no sulphur atom is present in D, for example, as a side group not in the direct covalent linkage between R^5 and A.

When R^1 and R^5 are a fluorescein/rhodamine pair there are preferably 9,10,11,12,13,14,15,16, or 17 linker atoms in A, and more preferably 9,10,11,12,13,14, or 15 linker atoms. When R^1 and R^5 are cyanine dyes, there are preferably 6,7,8,9,10,11,or 12 linker atoms, more preferably 8,9 or 10. Preferably A is a $C_{6,7,8,9,10,11,12,13,14,15,16,\text{ or }17}$ hydrocarbon chain.

The specification of a range of values for the number of atoms in a chain or group, whether an express listing of each integer within the range as above, or a description of the range by specifying the end points of the range, includes the specific description of each integer value within that range, including the endpoints. It further includes the specific description of each subrange within the larger range. For example, the range 1-6 includes the subranges 1-4 and 3-6, along with the other included subranges.

The reactive or functional group, R^3, may be any group suitable for attaching the energy transfer dye to a target material, preferably a target biological material and, as such, will be well known to those skilled in the art. Preferably R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite.

Suitable dyes for R^1 may be dyes which contain reactive or functional groups capable of linking with S. The attachment group D may be any group, other than sulphur, suitable for connecting R^5 with A. Preferably D is PO_3 or NH-CO.

The dye moieties, e.g., R^1 and R^5, of the present energy transfer dyes are fluorophores which are selected, as further indicated herein, to be able to participate in an energy transfer arrangement.

Preferably, the energy transfer dyes of this invention have a total molecular weight of less than 10,000 or 5,000 daltons, more preferably less than 3,000 or 2000 daltons, still more preferably less than 1,500 or 1,200 daltons.

In connection with the energy transfer dyes of the present invention, by "energy

transfer arrangement" is meant that two fluorescent dyes are selected having absorption and emission spectra suitable for energy transfer between the dyes, and located with sufficient physical proximity and linkage such that photoexitation of a first dye (the donor) results in the transfer of energy from the first dye to the second dye (the acceptor). Additional energy transfers involving one or more additional dye moieties can also be created.

Thus, an "energy transfer dye" refers to a fluroescent dye complex having at least two dye moieties which can participate in energy transfer between those two dye moieties.

By "acceptor" in an energy transfer arrangement is meant a dye moiety which absorbs energy at a wavelength emitted by a donor dye moiety, i.e., the absorption spectrum of the acceptor overlaps the emission spectrum of the donor.

By "donor" in an energy transfer arrangement is meant a dye moiety which absorbs energy from light, and emits light at frequencies at least partially within the absorption spectrum of an acceptor dye moiety.

By "linear or branched alkyl" is meant a straight-chain or branched saturated aliphatic hydrocarbon. Typical alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, pentyl, hexyl and the like. By halo is meant fluoro, chloro, bromo or iodo.

In the context of this invention, the term "target material" refers to a compound or structure to which a energy transfer dye is to be covalently attached or to which such a dye is attached.

By "biological material" is meant a compound produced by or present in an organism, including but not limited to polypeptides, nucleic acid molecules, carbohydrates, and lipids. Such compounds may be derivatised to include a group suitable for covalent attachment of an energy transfer dye. The term does not mean that the dyes of the present invention must be used with intact organisms, as often the dyes will be used with extracts, such as nucleic acid extracts, or samples, including preserved samples such as tissue sections, or in nucleic acid sequencing reactions.

Preferably, the energy transfer dye is of the formula (II):

$$R^1\text{-}S\text{-}(CHR^2)_m\text{-}CH(R^3)\text{-}NH\text{-}CO\text{-}(CHR^4)_n\text{-}NH\text{-}CO\text{-}R^5$$

(II)

wherein R^1 is a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^2 is hydrogen, $C_{1,2,3\text{ or }4}$ linear or branched alkyl or phenyl (optionally substituted as above);

R^3 is hydrogen or a reactive or functional group other than thiol;

R^4 is hydrogen, $C_{1,2,3\text{ or }4}$ linear or branched alkyl, or substituted or unsubstituted phenyl;

R^5 is a second dye that is suitable as a donor or acceptor in an energy transfer arrangement with the first dye;

m is 1,2 or 3;

n is 1,2,3,4,5,6,7,8, or 9.

Preferably, the donor dye is a fluorescein or cyanine dye. Preferably R^1 contains a reactive or functional group suitable for covalent attachment of the dye to a thiol-containing component of A. In the case of attachment to thiol groups, preferred reactive groups include iodoacetamido- and maleimido- groups.

Preferably the acceptor is a rhodamine or cyanine dye. Preferably R^5 contains a reactive or functional group suitable for attachment of the dye to a corresponding functional or reactive group component of A. For example, for attachment of R^5 to linker chain A which terminates in amino, dyes which contain a carboxyl or activated carboxyl group are preferred. The choice of reactive and functional group-containing dyes which are suitable for forming covalent linkages with the linker chain will be well known to those skilled in the art.

Suitable fluorescein donor dyes include but are not limited to 5- and 6-carboxyfluorescein and 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein.

Suitable cyanine donor dyes include but are not limited to CyA (3-(-carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), Cy3 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine).

Suitable rhodamine acceptor dyes include, but are not limited to 6-carboxyrhodamine (Rhodamine 110), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-

carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or TMR), 6-carboxy-X-rhodamine (ROX).

Suitable cyanine acceptor dyes include but are not limited to, Cy3.5 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)-dibenzo-dicarbocyanine, Cy7 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine. Cyanine dyes suitable for use in the energy transfer dyes of the present invention are disclosed in US Patent No. 4,268,486 (Waggoner et al; incorporated herein by reference in its totality including any drawings).

The above and additional dyes are described, for example, in Southwick et al., 1990, *Cytometry* 11:418-430; Mujumdar et al., 1993, *Bioconjugate Chemistry* 4:105-111; and Waggoner and Ernst, Fluorescent Reagents for Flow Cytometry, Part 1: Principles of Clinical Flow Cytometry (1993).

Optionally the complexes may contain a third dye, e.g. a cyanine dye, attached to R^5 through a suitable linker group and being in an energy transfer arrangement with R^5.

Suitably R^2 is hydrogen or methyl and preferably hydrogen.

It will be appreciated by those skilled in the art that when m is other than 1, there will be several R^2 groups present. In such a situation the R^2 groups may be the same or different. Preferably R^2 is hydrogen.

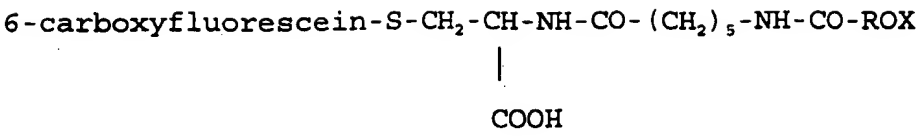
Similarly, R^4 is preferably hydrogen or methyl, preferably hydrogen. When n is other than 1 then the R^4 groups may be the same or different.

R^3 is as hereinbefore defined and is preferably a carboxyl or activated carboxyl group such as succinimidyl ester or sulpho-succinimidyl ester.

Suitably m + n is 7,8,9 or 10 when R^1/R^5 is a fluorescein/rhodamine pair and 3,4 or 5 when R^1 and R^5 are cyanine dyes.

Suitably m is 1,2 or 3, preferably 1. Suitably n is 1,2,3,4,5,6,7,8, or 9 and preferably 5 for a fluorescein/rhodamine pair.

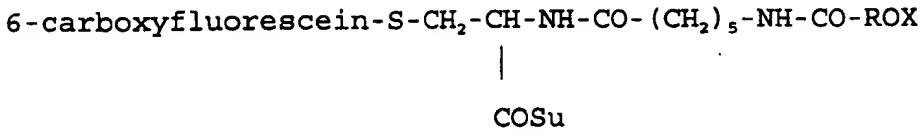
Preferred compounds of the present invention are:



6-carboxyfluorescein-S-CH_2-CH-NH-CO-$(CH_2)_5$-NH-CO-ROX

COOH

and



6-carboxyfluorescein-S-CH_2-CH-NH-CO-$(CH_2)_5$-NH-CO-ROX

COSu

where Su = N-hydroxysuccinimidyl

In a further aspect, the present invention relates to a biological material containing an energy transfer dye of the formula (I) or (II).

Suitable biological materials include, but are not limited to, antibodies, antigens, peptides, proteins, carbohydrates, lipids, nucleotides, oxy or deoxy polynucleic acids and cells which may be derivatised, if necessary so that they contain one or more groups suitable for attachment of an energy transfer dye, e.g., amino, hydroxy, thiophosphoryl, sulphydryl or carboxy groups.

In a further aspect, the present invention provides a method for the preparation of an energy transfer dye of the present invention using at least three coupling reactions:

(i) coupling the dye R^1 to a thiol containing component of A, which will also contain R^3, where A, R^1 and R^3 are as hereinbefore defined;

(ii) coupling the product of reaction (i) with the remaining part of A which will be substituted by a reactive group suitable for forming the attachment group, said D, and

(iii) coupling the product of reaction (ii) with the dye R^5 thereby forming the attachment group, said D.

In connection with the present energy transfer dyes and the attachment of the

various moieties of those dyes, the term "coupling" refers to the formation of a covalent bond(s) linking two components, for example, linking a dye moiety with the A portion of the energy transfer dye.

As there may be several reactive groups present in any component taking part in one of the coupling reactions, it may be necessary for those not taking part in that reaction to be blocked or protected and then deprotected as appropriate later in the reaction sequence.

The dye R^1 will normally contain a substituent suitable for reaction with a thiol group or will be modified to contain such a group. For example, iodoacetamide is a suitable substituent for fluorescein dyes, maleimido is a suitable substituent for cyanine dyes.

The fluorescent labeling energy transfer dyes may be used to form reagents by covalently binding the dyes to carrier materials such as polymer particles, cells, glass beads, antibodies, proteins, peptides, enzymes, carbohydrates, lipids and nucleotides or nucleic acids (DNA and RNA) and analogues which contain or have been derivatised to include at least one first reactive group capable of forming a covalent bond with the functional group on the labeling complex (or functional group capable of forming a covalent bond with a reactive group on the complex, as described above) and at least one second reactive group (or functional group, as the case may be), having specificity for, and being capable of forming a covalent bond with, a target biological compound, such as antibodies, cells, drugs, antigens, bacteria, viruses and other micro-organisms.

When the carrier has functional groups, said functional groups may be antibody or DNA suited for attachment to antigen or a complementary DNA sequence, respectively. When the carrier material has reactive groups, the carrier may be a polymer particle or an antigen suitable for attachment to DNA or an antibody for example. Techniques for covalently binding fluorochromes to carrier materials such as those mentioned are well known in the art and readily available in the literature.

The carrier material can further include nucleotides derivatised to contain one of amino, sulphydryl, carboxyl, carbonyl or hydroxyl groups, and oxy or deoxy polynucleic acids derivatised to contain one of amino, thiophosphoryl, sulphydryl, carboxyl, carbonyl or hydroxyl groups.

The functional groups on the carrier material which are complementary to i.e. capable of forming covalent bonds with, the reactive groups of the labeling complexes of the invention include amino, sulphydryl, carboxyl, carbonyl and hydroxyl groups.

The present invention also relates to labeling processes in which, in a first step, an energy transfer dye of the present invention covalently reacts with and thereby labels a first component and then uses the labeled first component to bind with a second component to form a labeled second component. Suitably, the first component may be one member of a specific binding pair, (a specific binding partner). In the second step of the procedure, the fluorescently labeled specific binding partner is then used as a probe for binding to a second member of the specific binding pair (the second component) for which it has specific affinity.

The specific binding pairs may include a wide variety of molecules which are arbitrarily termed ligands and receptors. An example of such ligand-receptor pairs includes an antibody and the corresponding antigen for which the antibody is specific. When the target of the so-labeled antibody is a cell, the second step of the procedure may be used to determine the amount of labeled antibodies which are attached to that type of cell by determining the intensity of the fluorescence of the cells. By this procedure, monoclonal antibodies and other components covalently labeled in a first step with the fluorescent compounds of the present invention could be used as antigen probes.

Numerous other examples are known to those skilled in the art. Thus, additional ligand-receptor pairs include, for example, biotin-(strept)avidin, hormone receptor-hormone, DNA-complementary DNA, DNA-RNA, DNA-binding protein, enzyme-substrate, and the like. It is to be understood that any two molecules which possess a specific binding affinity may be employed, so that the energy transfer dyes of the present invention may be used for labeling one member of a specific binding pair which in turn may be used in the detection of the complementary member.

The energy transfer dyes of the present invention provide a valuable set of fluorescent labels which are particularly useful for multiparameter analysis and importantly, are sufficiently low in molecular weight to permit materials labeled with the fluorescent complexes to penetrate cell structures. As such, the dyes are well suited for use with DNA probes. Multiparameter analysis can be performed on multiple samples to

detect the presence of target biological compounds. Each sample is labeled by well known labeling methods with a different dye or energy transfer dye.

For example, one sample suspected of containing a target biological compound is incubated with a single fluorochrome, such as fluorescein, Cascade Blue, a BODIPY dye, or one of the monomethine rigidized dyes, or CY3O$(SO_3)_2$, or CY3$(SO_3)_2$, all emitting in the 500-575nm wavelength range (green to orange). A second sample suspected of containing the target biological compound (the same compound or a different compound as that in sample 1), is incubated with an energy transfer dye of the invention, for example fluorescein-CY3NH_2, which will absorb light at 488nm and emits fluorescence at 574nm (orange). Additional samples suspected of containing another target compound are incubated with other dyes of the invention, such as fluorescein-CY3-CY5 and fluorescein-CY3-CY7, both of which absorb light at 488nm, but emit fluorescence at 672nm and 782nm respectively (red to near infra-red). After a suitable period to permit the fluorescent labels to bind with the target compounds, unbound label is removed by washing and the labeled samples are mixed.

Detection is possible with a single wavelength excitation source, i.e. at laser line 488nm. Each differently labeled sample will fluoresce a different color at the emission wavelength of its particular label, allowing the individual labels to be distinguished from each other.

Those skilled in the art will recognize that the fluorescent energy transfer labeling dyes of the present invention can be used for a variety of immunofluorescent techniques, including direct and indirect immunoassays, and other known fluorescent detection methods. The conditions of each labeling reaction, e.g. pH, temperature and time are known in the art, but generally room temperature is preferred. If reacting with an amine, pH 9.4 is preferred. The pH is adjusted depending on the optimum reaction conditions for the particular reactive groups according to known techniques.

The energy transfer dyes of the present invention and the reagents that can be made from them offer a wide variety of fluorescent labels with large Stokes' shifts. Those in the art will recognize that the dyes of the invention can be used in a variety of fluorescence applications over a wide range of the visible spectrum.

Other features and advantages of the invention will be apparent from the following

description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The following examples serve to illustrate the preparation of the energy transfer dyes of the present invention. These examples are in no way intended to limit the scope of the invention.

Example 1

Preparation of FAM-Cysteine-Linker-ROX Energy Transfer Dye

i) FAM-Cysteine Coupling Reaction

The following mixture was prepared in a microcentrifuge tube: 300 l of 0.25M L-cysteine (free base), 200 l 1M potassium phosphate, pH 8, 800 l of a solution of 50mg/ml 5-iodoacetamido-fluorescein (Molecular Probes) in DMF, and 700 l of water. The reaction mixture was incubated at room temperature for 1 hour protected from light.

ii) FAM-Cysteine-Linker Coupling Reaction

To the crude reaction mixture from i) was added 600 l water, 400 l 1M sodium carbonate buffer, pH 8.3, and 1ml of a solution of 75mg/ml of the trifluoroacetyl-protected NHS ester of 6-aminocaproic acid in DMF. The reaction mixture was incubated at room temperature for 1 hour protected from light.

iii) Deprotection of the FAM-Cysteine-Linker

The reaction mixture from ii) was dried in a Speed-Vac apparatus and 10ml of concentrated ammonium hydroxide was added to the tube. After thorough mixing, the reaction was incubated at room temperature for 2 hours and then dried in the Speed-Vac apparatus.

iv) Purification of FAM-Cysteine Linker

Purification was accomplished by HPLC using a DeltaPak column (15 micron, C18

reverse phase, 7.8 x 300mm) and a gradient of triethylammonium acetate, pH 7.0 and 50% acetonitrile in triethylammonium acetate, pH 7.0. Fractions with absorbance at 496nm were collected and dried overnight in a SpeedVac apparatus.

v) FAM-Cysteine-Linker-ROX Coupling Reaction

To a microcentrifuge tube was added the following:

150µl HPLC purified FAM-Cysteine-Linker in DMF equivalent to 3mg (as measured by absorbance at 496nm), 100µl water, 50µl 1M sodium carbonate buffer, pH 8.3, and 200µl 5'-ROX-NHS ester (Molecular Probes) (25mg/ml in DMF). The reaction mixture was incubated overnight at room temperature.

Note: Other rhodamine acceptor dyes may be substituted for ROX in the above reaction to generate four different energy transfer dye cassettes for use in sequencing applications.

vi) Purification of FAM-Cysteine-Linker-ROX

The product energy transfer dye from v) was purified by reverse phase HPLC using a DeltaPak column (15 micron, 300A, C18 reverse phase, 7.8 x 300mm) and a gradient of triethylammonium acetate, pH 7.0 and 50% acetonitrile in triethylammonium acetate, pH 7.0. Fractions having absorbance at both 496nm and 576nm were collected, pooled and dried overnight in a SpeedVac apparatus. The product was redissolved in water and evaluated spectroscopically using a Perkin Elmer LS-50B Luminescence Spectrometer. With excitation at 488nm, a strong peak was observed at 603nm, characteristic of the ROX emission and indicating excellent energy transfer. The absorbance spectrum showed bands characteristic of both fluorescein and ROX.

vii) Conversion of the Carboxylic Acid Derivative to its NHS Ester

The cysteine carboxyl of the above energy transfer dye may be converted to an NHS ester derivative by the following method.

The above acid derivative is dissolved in DMF at a concentration of 10mg/ml. To the stirred solution in a round bottomed flask is added 1.5 mole equivalents of

dicyclohexylcarbodiimide (DCC) and 1.5 mole equivalents of N-hydroxysuccinimide. The flask containing the mixture is sealed and covered with foil. The reaction is stirred under argon at room temperature for 4 hours. It is then checked by TLC using the appropriate standards to monitor that the free acid has been converted into the NHS ester (TLC using dichloromethane-methanol-acetic acid, 4:1:1). The activated dye is then precipitated from solution by adding four volumes of ethyl acetate. The resulting pellet is rinsed 2 times with ethyl acetate and finally dried under vacuum. The NHS ester may now be coupled to a suitable target material (e.g. an amino-link oligonucleotide) using standard conditions.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The dyes, substituents, and target materials described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. For example, those skilled in the art will readily recognize that the present energy transfer dyes can incorporate a variety of different dye moieties, linkers, attachment groups, and reactive groups, and can be attached to a variety of different target materials. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Thus, additional embodiments are within the scope of the invention and within the following claims.

Claims

What we claim is:

1. An energy transfer dye of the formula:

$$R^1\text{-}S\text{-}A\text{-}D\text{-}R^5 \quad (R^3 \text{ attached to A})$$

wherein

R^1 is a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 is a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linearly linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 is a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does not include a sulphur atom.

2. The energy transfer dye of claim 1, wherein

said A-D is $(CHR^2)_m$-$CH(R^3)$-NH-CO-$(CHR^4)_n$-NH-CO,

wherein

R^2 is hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

R^3 does not consist of thiol;

R^4 is hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

m is 1,2, or 3; and

n is 1,2,3,4,5,6,7,8, or 9.

3. The energy transfer dye of claim 1, wherein said A comprises a C_6-C_{17} hydrocarbon chain.

4. The energy transfer dye of claim 1, wherein said R^1 is selected from the group consisting of a fluorescein dye and a cyanine dye, and said R^5 is selected from the group consisting of a rhodamine dye and a cyanine dye.

5. The energy transfer dye of claim 4, wherein said R^1 is selected from the group consisting of:
5-carboxyfluorescein, 6-carboxyfluorescein, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, CyA (3-(-carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), and Cy3 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine).

6. The energy transfer dye of claim 4, wherein said R^5 is selected from the group consisting of:
6-carboxyrhodamine (Rhodamine 110), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or TMR), 6-carboxy-X-rhodamine (ROX), Cy3.5 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)-dibenzo-dicarbocyanine, and Cy7 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine.

7. The energy transfer dye of claim 1, wherein said target material comprises a biological material.

8. The energy transfer dye of claim 1, wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

9. The energy transfer dye of claim 4, wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

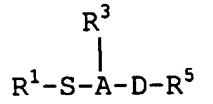
10. The energy transfer dye of claim 1, further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

11. The energy transfer dye of claim 4, further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement or said third dye with said R^5.

12. The energy transfer dye of claim 10, wherein said third dye is a cyanine dye.

13. The energy transfer dye of claim 11, wherein said third dye is a cyanine dye.

14. A method for producing an energy transfer dye of the formula:



wherein

R^1 is a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 is a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 comprises a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does not include a sulphur atom;

said method comprising:

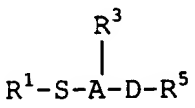
(a) coupling said R^1 to a thiol containing component of said A, wherein said A also comprises said R^3;

(b) coupling the product of part (a) with the remaining part of said A, wherein said remaining part of A is substituted by a reactive group suitable for forming the attachment group D, and

(c) coupling the product of (b) with said R^5 thereby forming said attachment group D.

15. A method for fluorescently labeling a biological material comprising:

a) adding to a liquid which contains said biological material an energy transfer dye of the formula:



wherein R^1 comprises a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 comprises a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 comprises a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does include a sulphur atom; and

b) reacting said dye so that said dye covalently reacts with and labels said biological material.

16. The method of claim 15, wherein said biological material is selected from the group consisting of antibodies, antigens, peptides, proteins, carbohydrates, lipids, nucleotides,

oxy or deoxy polynucleic acids and cells which are optionally derivatised so that they contain one or more amino, hydroxy, thiophosphoryl, sulphydryl or carboxy groups.

17. The method of claim 15, wherein said energy transfer dye comprises a compound, wherein

said A-D is $(CHR^2)_m$-$CH(R^3)$-NH-CO-$(CHR^4)_n$-NH-CO;

R^2 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

R^3 does not consist of thiol;

R^4 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

m is 1,2, or 3; and

n is 1,2,3,4,5,6,7,8, or 9.

18. The method of claim 15, wherein said energy transfer dye comprises a compound wherein said A comprises a C_6-C_{17} hydrocarbon chain.

19. The method of claim 15, wherein said energy transfer dye comprises a compound wherein said R^1 is selected from the group consisting of a fluorescein dye and a cyanine dye, and said R^5 is selected from the group consisting of a rhodamine dye and a cyanine dye.

20. The method of claim 19, wherein said energy transfer dye comprises a compound wherein said R^1 is selected from the group consisting of:

5-carboxyfluorescein, 6-carboxyfluorescein, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, CyA (3-(-carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), and Cy3 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine).

21. The method of claim 19, wherein said energy transfer dye comprises a compound wherein said R^5 is selected from the group consisting of:

6-carboxyrhodamine (Rhodamine 110), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-6-carboxyrhodamine

(TAMRA or TMR), 6-carboxy-X-rhodamine (ROX), Cy3.5 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)-dibenzo-dicarbocyanine, and Cy7 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine.

22. The method of claim 15, wherein said energy transfer dye comprises a compound wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

23. The method of claim 19, wherein said energy transfer dye comprises a compound wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

24. The method of claim 15, wherein said energy transfer dye comprises a compound further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

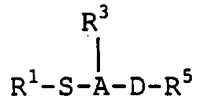
25. The method of claim 19, wherein said energy transfer dye comprises a compound further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

26. The method of claim 25, wherein said third dye is a cyanine dye.

27. The method of claim 24, wherein said third dye is a cyanine dye.

28. A method for fluorescently labeling a first component and then using said labeled first component to detect the presence of a second component in a sample, comprising:

a) adding to a liquid which contains said first component an energy transfer dye of the formula:



wherein R^1 comprises a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 comprises a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 comprises a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does include a sulphur atom;

b) reacting said dye with said first component such that said dye covalently reacts with and thereby labels said first component;

c) providing said labeled first component to said sample to permit binding of said labeled first component to said second component if present; and

d) detecting said second component if present by detecting the fluorescent label by an optical method.

29. The method of claim 28, wherein said energy transfer dye comprises a compound wherein

said A-D is $(CHR^2)_m$-$CH(R^3)$-NH-CO-$(CHR^4)_n$-NH-CO, wherein

R^2 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

R^3 does not consist of thiol;

R^4 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

m is 1,2, or 3; and

n is 1,2,3,4,5,6,7,8, or 9.

30. The method of claim 28, wherein said energy transfer dye comprises a compound wherein said A comprises a C_6-C_{17} hydrocarbon chain.

31. The method of claim 28, wherein said energy transfer dye comprises a compound wherein said R^1 is selected from the group comprising a fluorescein dye and a cyanine dye, and said R^5 is selected from the group comprising a rhodamine dye and a cyanine dye.

32. The method of claim 31, wherein said energy transfer dye comprises a compound wherein said R^1 is selected from the group consisting of: 5-carboxyfluorescein, 6-carboxyfluorescein, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, CyA (3-(-carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), and Cy3 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine).

33. The method of claim 31, wherein said energy transfer dye comprises a compound wherein said R^5 is selected from the group consisting of: 6-carboxyrhodamine (Rhodamine 110), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or TMR), 6-carboxy-X-rhodamine (ROX), Cy3.5 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)-dibenzo-dicarbocyanine, and Cy7 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine.

34. The method of claim 28, wherein said target material comprises a biological material.

35. The method of claim 28, wherein said energy transfer dye comprises a compound wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester,

sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

36. The method of claim 31, wherein said energy transfer dye comprises a compound wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

37. The method of claim 28, wherein said energy transfer dye comprises a compound further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

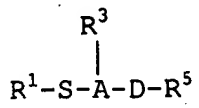
38. The method of claim 31, wherein said energy transfer dye comprises a compound further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

39. The method of claim 37, wherein said third dye is a cyanine dye.

40. The method of claim 38, wherein said third dye is a cyanine dye.

41. A reagent comprising:

a) an energy transfer dye of the formula:



wherein R^1 comprises a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 comprises a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 comprises a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does include a sulphur atom; and

b) a carrier material which contains or has been derivatised to include at least one first reactive group capable of forming a covalent bond with a functional group, or functional group capable of forming a covalent bond with a reactive group on the energy transfer dye and is covalently bonded thereto.

42. The reagent of claim 41, wherein said carrier material is selected from the group consisting of polymer particles, cells, glass beads, antibodies, proteins, peptides, enzymes, carbohydrates, lipids and nucleic acids.

43. The reagent of claim 42, wherein said energy transfer dye comprises a compound, wherein

said A-D is $(CHR^2)_m$-$CH(R^3)$-NH-CO-$(CHR^4)_n$-NH-CO, wherein

R^2 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

R^3 does not consist of thiol;

R^4 comprises hydrogen, a 1,2,3, or 4 carbon containing alkyl or phenyl;

m is 1,2, or 3; and

n is 1,2,3,4,5,6,7,8, or 9.

44. The reagent of claim 42, wherein said energy transfer dye comprises a compound wherein said A comprises a C_6-C_{17} hydrocarbon chain.

45. The reagent of claim 41, wherein said energy transfer dye comprises a compound wherein said R^1 is selected from the group comprising a fluorescein dye and a cyanine dye, and said R^5 is selected from the group comprising a rhodamine dye and a cyanine dye.

46. The reagent of claim 45, wherein said energy transfer dye comprises a compound, wherein said R^1 is selected from the group consisting of: 5-carboxyfluorescein, 6-carboxyfluorescein, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, CyA (3-(-carboxypentyl)-3'-ethyl-5,5'-dimethyl oxacarbocyanine), and Cy3 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-carbocyanine).

47. The reagent of claim 45, wherein said energy transfer dye comprises a compound wherein said R^5 is selected from the group consisting of: 6-carboxyrhodamine (Rhodamine 110), 5-carboxyrhodamine-6G (R6G-5 or REG-5), 6-carboxyrhodamine-6G (R6G-6 or REG-6), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or TMR), 6-carboxy-X-rhodamine (ROX), Cy3.5 (3-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)dibenzo-carbocyanine), Cy5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-dicarbocyanine, Cy5.5 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-4,5,4',5'-(1,3-disulphonato)-dibenzo-dicarbocyanine, and Cy7 (1-(-carboxypentyl)-1'-ethyl-3,3,3',3'-tetramethyl-5,5'-disulphonato-tricarbocyanine.

48. The reagent of claim 41, wherein said target material comprises biological material.

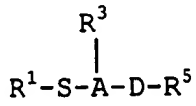
49. The reagent of claim 41, wherein said energy transfer dye comprises a compound wherein said R^3 is selected from the group consisting of carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide and phosphoramidite, and groups covalently reactive with carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, and phosphoramidite.

50. The reagent of claim 41, wherein said energy transfer dye comprises a compound further comprising a third dye attached to said R^5 through a linker group, wherein said attachment results in an energy transfer arrangement of said third dye with said R^5.

51. The reagent of claim 50, wherein said third dye is a cyanine dye.

52. A biological material with an attached energy transfer dye, comprising:

a) an energy transfer dye of the formula:



wherein R^1 comprises a first dye suitable as an acceptor or donor in an energy transfer arrangement;

R^5 comprises a second dye suitable as a donor or acceptor in an energy transfer arrangement with said first dye;

A comprises a chain of 5 to 20 linked atoms, wherein each atom is independently selected from the group consisting of carbon, nitrogen and oxygen;

R^3 comprises a hydrogen or a reactive or functional group suitable for attaching the energy transfer dye to a target material; and

D comprises an atom or group for attaching said R^5 to said A, wherein the covalent linkage between said A and said R^5 does include a sulphur atom; and

b) a biological material which includes or has been derivatised to include at least one first reactive group capable of forming a covalent bond with a functional group, or a functional group capable of forming a covalent bond with a reactive group on the energy transfer dye and which is covalently bonded thereto.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02105

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/546; 435/6, 7.2, 188; 436/527, 531, 800; 530/391.5, 408; 549/223, 227

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE
search terms: energy transfer dye, rhodamine, fluorescein, carbocyanine, CyA, Cy3, Cy5, Cy5.5, Cy7, fluorescent labeling

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- X,Y	US 5,853,992 A (GLAZER et al) 29 December 1998, col. 4, lines 29-37; col. 4, line 62 - col. 5, line 20, in particular "polypeptides" as linkers; col. 7, line 65 - col. 8, line 27.	1-13 ---- 14-52
X,P ---- Y,P	US 5,814,454 A (JU) 29 September 1998, col. 5, line 54 - col. 6, line 29; col. 6, line 56 - col. 7, line 9; col. 8, lines 1-24.	1-13 ---- 14-52
X,P ---- Y,P	US 5,800,996 A (LEE et al) 01 September 1998, col. 3, line 60 - col. 4, line 15; col. 5, line 8 - col. 6, line 59.	1-13 ---- 14-52
X,P ---- Y,P	US 5,728,528 A (MATHIES et al) 17 March 1998, col. 3, line 66 - col. 4, line 33.	1-13 ---- 14-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 APRIL 1999	Date of mailing of the international search report 20 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY E. CEPERLEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02105

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,654,419 A (MATHIES et al) 05 August 1997, col. 9, lines 35-51.	1-13 ---- 14-52
X -- Y	LEE. L.G. et al. New energy transfer dyes for DNA sequencing. Nucleic Acids Research. 1997, Vol. 25, No. 14, pages 2816-2822, especially page 2817, preparation of fluorescein-rhodamine dimers.	1-13 ---- 14-52
X -- Y	EP 805,190 A2 (PERKIN-ELMER CORPORATION) 02 May 1997, especially Summary of the Invention of page 3.	1-13 ---- 14-52
X -- Y	US 5,707,804 A (MATHIES et al) 13 January 1998, col. 4, lines 1-25.	1-13 ---- 14-52

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02105

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/533; C07D 311/82, 311/88; C07K 1600; C12N 9/96; GO1N 33/52, 33/533, 33/545, 33/548, 33/552, 33/554

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/546; 435/6, 7.2, 188; 436/527, 531, 800; 530/391.5, 408; 549/223, 227